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The crypt cycle in mouse small intestinal epithelium

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SUMMARY

We have used a mutation-induced marker system in the intestine of mice heterozygous at the *Dlb-1* locus, which determines the expression of binding sites for the lectin *Dolichos biflorus* agglutinin, and the frequency of clustering of mutated crypts with time as a means of investigating the frequency of the crypt fission process and the crypt cycle. Whole-mount preparations from heterozygous *Dlb-1^b/Dlb-1^a* mice were stained with a peroxidase conjugate of *Dolichos biflorus* agglutinin. Mutations at the *Dlb-1^b* locus in crypt stem cells result in loss of DBA-Px binding in these cells and subsequently their progeny, which eventually results in a rare isolated single, unstained crypt. The subsequent development of pairs, triplets and clusters of negative staining crypts has been assumed to be the result of crypt fission. The frequency of these fission events has been measured in control untreated mice. These negative crypts are the result of spontaneous mutations. We have also looked at mutated crypts after treatment with *N*-nitroso-*N*-ethylurea or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine of young adult mice, which elevates the number of mutations.

Our results suggest that the crypt cycle in control animals is very long, 187±44 weeks (3.6 years, i.e. essentially the life of a laboratory mouse). This implies that about a third of the crypts may divide once in the life of a mouse. After sufficient time for conversion of mixed crypts to monophenotypic crypts after mutagen treatment several clusters of negative crypts were seen. This suggests that agents like *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine cause sufficient damage to actually destroy some crypts, which is confirmed by crypt survival studies, and this induces local crypt repopulation and fission.

Key words: somatic cell mutations, mouse small intestine, crypt fission, crypt cycle, chemical mutagen, *Dolichos biflorus* agglutinin binding

INTRODUCTION

In the mouse small intestine there are approximately 1.1 million organised groups of proliferating cells, the crypts (Hagemann et al., 1970), each of which contains about 250 cells (Kellett et al., 1992). The crypt contains 16 cells in circumference and about 20-25 cells in column height. Most of these cells are columnar, but there are some goblet, Paneth and entero-endocrine cells. Between 50 and 80% of these cells are proliferative with about 150-160 cells in the crypt as a whole being in rapid cycle. These rapidly cycling cells are located in a broad band across the middle of the crypt (Potten and Loeffler, 1990; Potten and Hendry, 1983). The crypt contains between 4 and 16 actual stem cells (most probably 4) with cell cycle times between 12 and 32 hours; average 24 hours (Potten and Loeffler, 1990). There are between 4 and 6 transit cell generations. The stem cells are most likely to be arranged within the annulus at about the 4th cell position from the crypt base. Each crypt produces about 12 cells per hour or about 300 new cells per day, which migrate to the villus surface with a velocity of about 0.75 cell position or cell diameter per hour (Potten and Loeffler, 1990). However, this migration exhibits strong circadian rhythm (Qiu et al., unpublished data). Each villus has between 6 and 14 crypts arranged around its base, the number varying with position along the intestine and each crypt usually serves two villi (Potten et al., 1982).

As there are several stem cells in each crypt, regeneration of the crypt is possible provided not all stem cells are reproductively sterilised. However, at high doses of a cytotoxic agent like radiation some crypts will contain no surviving clonogenic cells (Hendry and Potten, 1974). However, any surviving stem cells will repopulate the crypt and the epithelium by cell division and then crypt fission (Cairnie and Millen, 1975). This process can be studied quantitatively using the crypt microcolony assay technique (Potten and Hendry, 1985). At even higher doses where only the occasional crypt regenerates, these will eventually produce a localised region of regenerated epithelium, and a cluster of crypts. These foci of regeneration, when examined after 14 days, contain many crypts, which probably arose from a single surviving stem or regenerative cell in one crypt. These regenerating foci with many crypts may arise by a sequence of many binary fissions or more

probably by splitting simultaneously into many crypts (multiple fission). It is not known which cells in the crypt are able to contribute to the fission process but it is assumed to be the stem cells. The turnover time for crypt fission is not fully understood due to the limitations of the experimental techniques.

We have studied crypt fission in the normal small intestinal epithelium of C57BL/6J x SWR F1 heterozygous mice (*Dlb-1^b/Dlb-1^a*) and after treatment with the mutagens, i.e. *N*-nitroso-*N*-ethylurea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NEU, MNNG), using the lectin *Dolichos biflorus* agglutinin (DBA) as a strain-specific histochemical marker. This assay is a specific locus test for somatic mutations (Winton et al., 1988). The *Dlb-1* locus, on mouse chromosome 11 (Uiterdijk et al., 1986), determines the tissue-specific pattern of expression of the binding site for the DBA lectin. Mice homozygous for *Dlb-1^b*, e.g. C57BL/6J, express the binding site on all cells in the intestinal epithelium. Homozygous *Dlb-1^a* mice, e.g. SWR, display the converse pattern of expression with no binding to the epithelium but binding to some stromal elements such as endothelium (Ponder and Wilkinson, 1983). DBA binding is co-dominant, and in heterozygous *Dlb-1^a/Dlb-1^b* mice only the single *Dlb-1^b* allele specifies the lectin binding to intestinal epithelium. Mutations affecting this allele in a crypt stem cell can result, given time, in a clone of cells (initially in the crypt) that cannot bind DBA, which thus can be recognised as a white, negative-staining crypt in whole-mount preparations stained with a peroxidase conjugate of DBA (DBA-Px) and subsequently as a ribbon of negative cells on the villus. Singles, pairs, triplets and clusters of negative-staining crypts were scored at various ages and times after treatment, and the changes in the frequency of these categories were used as indications of crypt fission. Our results show that each crypt that contains a mutated stem cell will ultimately become monophenotypic, i.e. uniformly unstained (although the mechanisms involved here are unclear), and that the time needed for this to be complete is about 7 weeks. The interval of time between successive buddings or fissions of a crypt is 187±44 weeks in control mice. This may be accelerated soon after cytotoxic damage.

MATERIALS AND METHODS

Chemicals

NEU (Sigma) and MNNG (Sigma) were dissolved firstly in dimethylsulphoxide (DMSO) and then diluted with sterile saline (9 g l^{-1}) to give a concentration 10 mg/ml for NEU and 2.5 mg/ml for MNNG.

Animals

C57BL/6J × SWR F1 mice were bred from C57BL/6J (*Dlb-1^b/Dlb-1^b*, Paterson Institute) and SWR (*Dlb-1^a/Dlb-1^a*, Harlan Olac Ltd, UK). F1 male mice were used at 12 weeks of age in three experiments and F1 female mice were used when one year old in one experiment. Each group consisted initially of four animals, in three groups one animal died during the experiment. All the animals were housed under conventional conditions with a 12 hour, light/dark, cycle (lights on at 0600 hours). Food (ERM maintenance diet, SDS, Witham, Essex) and water were provided ad libitum. The animals were given an injection of either 50 mg MNNG/kg or 100 mg NEU/kg body weight intraperitoneally at the commencement of the experiment. The control groups were completely untreated (no vehicles). Groups of animals were killed at various times after treatment (up to 52 weeks for MNNG, 86 weeks for NEU, and 136 weeks for controls).

Preparation of tissues and lectin staining

The method used for crypt staining is based on that of Winton and Ponder (1990). The animals were killed by cervical dislocation. A 5 cm segment of distal small intestine was removed, placed into 0.9% saline, cut along the mesenteric side, and pinned out on a wax-based dish using entomological pins with the luminal (villus) side upmost. Following fixation for 30 minutes in 10% formol-saline, the preparations were rinsed and covered with a mucus-removing solution (200 ml absolute ethanol, 100 ml glycerol, 100 ml 0.1 M Tris-HCl, pH 8.2, 600 ml 1% saline) for 30 minutes. They were then covered with phosphate buffered saline (PBS, Unipath Ltd, UK) containing 0.5% bovine serum albumin (BSA, Sigma) for 30 minutes. To block endogenous peroxidase activity, the small intestinal sheets were incubated for 30 minutes in a methanol:hydrogen peroxide solution (40:0.2, v/v). Following two washes in the PBS/BSA solution, the sheets were incubated overnight in DBA-Px (Sigma) at a concentration 7.5 μg/ml in PBS/BSA. The peroxidase activity was subsequently demonstrated using 3',3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a substrate yielding a brown reaction product (Ponder and Wilkinson, 1983). A 20 mg sample of DAB was dissolved in 40 ml PBS at pH 7.6 and 30 μl of 30% hydrogen peroxide was added. The intestinal sheets were incubated for 10 minutes in the DAB solution. The preparations were then turned over and repinned with the abluminal (crypt) side upmost. They were refixed in methanol:glacial acetic acid (3:1, v/v) for 30 minutes before being transferred to 70% ethanol. This made the stripping of the muscle layers from the remainder of the epithelium easier (Winton et al., 1988). The preparations then underwent a second DBA-Px staining and DBA development as above.

Quantification of DAB-Px negative staining crypt

The preparations were examined for DBA-Px negative staining crypts starting at one end of the intestine using a Zeiss microscope, using a ×20 objective and square fields defined by an eyepiece grid. Negative events were recognized as white unstained crypts against the brown background staining (see Figs 1,2). Singles, pairs, triplets and clusters of negative staining crypts were recorded as well as two negative crypts one, or two, normal crypts apart. The field being scored was temporarily moved if a negative crypt appeared at the edge to see if there was a second negative crypt in an adjacent field. Three counts were made of the total number of crypts in the first, the middle and the last grid analysed and the total number of crypts scored was estimated from the mean of these three observations and the total number of grids analysed. For each animal the number of negative crypts N_t (various type) scored was corrected to 10^5 crypts using the following formula:

$$N_t = [N_e \times 10^5]/[M_n \times L \times W],$$

where N_e is the number of negative events scored from each preparation; M_n is the mean number of crypts from three grids; and L and W represent the length and width of the whole gut preparation (measured as units of the grid), respectively. Usually about 3,500 grids (magnification ×250) were screened in this way from each of the three to four mice in a group and each grid contained about 20-30 crypts. Thus between 70,000 and 105,000 crypts were generally screened per mouse.

Since multiple budding crypts have been seen in the adult mouse after irradiation (Cairnie and Millen, 1975) but they are rare in untreated mice, it is likely that any large clusters seen in the present experiment represent foci of crypt repopulation after the damage induced by the treatment (chemical mutagens) or mutations that occurred during embryogenesis. Here, pairs, triplets and clusters (excluding any with more than 20 negative crypts in the cluster, which may be formed during embryogenesis) have been used to assess the

rate of crypt division, i.e. the crypt cycle (Totafurno et al., 1987). In order to derive estimates of the spontaneous mutation rate (α_m), the rate at which mutated crypts are converted to monoclonality (α_c) and the crypt fission rate (α_f), the simplified compartmental model described in the Appendix is adopted. In the model mutations occurring in crypts will give mixed crypts that convert to monoclonality at a constant rate. These single monoclonal crypts then undergo fission at a constant rate to become clusters.

In the Appendix equations describing the number of single mutated crypts ($S(t)$) and the number of clusters ($C(t)$) as a function of time are derived for the two situations of interest.

(1) Untreated animals:

$$S(t) = (\alpha_m/\alpha_f)\,(1-\exp(-\alpha_f t));$$
$$C(t) = \alpha_m t + (\alpha_m/\alpha_f)\,(\exp(-\alpha_f t)-1). \quad (1)$$

(2) Mutagen-treated animals:

$$S(t) = M_0\,\alpha_c\,(\exp(-\alpha_f t)-\exp(-\alpha_c t))/(\alpha_c-\alpha_f)$$
$$C(t) = M_0\,\{1-(\alpha_c\exp(-\alpha_f t)-\alpha_f\exp(-\alpha_c t))/(\alpha_c-\alpha_f)\}, \quad (2)$$

where M_0 is the number of mutated crypts induced at the end of the mutagen treatment. The conversion and fission rates can be converted to conversion and fission times using the relation: $t = \ln(2)/\alpha$.

An unweighed least squares algorithm was used to fit equation (1) to the control data and equation (2) to each set of data from mutagen-treated animals. All sizes of clusters were included in the fitting of $C(t)$, including those one or two crypts apart: i.e. $C(t)$ was fitted to the data in column J of Table 1. Each animal was considered as a separate unit in the fitting.

In order to determine whether MNNG was sterilising crypts a small-scale dose-response experiment was performed using the crypt microcolony assay (Withers and Elkind, 1970; Hendry and Potten, 1974). Briefly, a range of MNNG doses was used, groups of animals were killed three days later and haematoxylin- and eosin-stained transverse sections of the intestine were cut. The number of surviving (regenerating) colonies was determined by counting 10 cross-sections from each of the four mice in a group.

RESULTS

The whole-mount preparations were examined from the crypt side and unmutated (wild-type) crypts appear as circular structures stained brown by the DBA-Px conjugate, while mutated (negative) crypts can be seen as white circular areas with the cellular outlines visible (Figs 1, 2). The mutated crypts were divided into eight categories (Table 1): (A) isolated or single completely negative crypts, which show no DBA-Px staining at all (Figs 1B, 2A); (B) single half-crypts, which have a sharp delineation between a segment of positive- and negative-staining epithelium: the relative proportions of each component being variable from crypt to crypt; (C) negative crypts with positive Paneth cells; these are negative-staining crypts with a few (between 1 and 12) positive-staining Paneth-like cells containing numerous refractile granules situated towards the crypt base (Fig. 1A); the appearance of these positive-staining cells is consistent with the published descriptions of the non-proliferative Paneth cells (Winton and Ponder, 1990; Cheng, 1974); (D) paired negative crypts; one negative staining crypt immediately adjacent to another (wholly negative, or negative crypts with positive Paneth cells, are considered together) (Fig. 1C); (E) triplets of negative crypts; a similar category to D, but with three negative staining crypts together (Fig. 1D); (F) clusters of negative crypts; more than three negative staining crypts forming a cluster (Fig. 2B,C); (G) two negative crypts one positive crypt apart; (H) two negative crypts two positive crypts apart. The numbers of all categories have been normalised to 10^5 crypts in Table 1.

The numbers of paired, triplet, clustered and paired (either one or two positive crypts apart) crypts was taken as indications of fission of mutated (i.e. budded at least once) crypts. The total number of these could be used to estimate the crypt cycle time. The data for untreated control mice (4 weeks to 136 weeks) and for the treated groups (NEU, 2 weeks to 86 weeks; MNNG, 2 weeks to 52 weeks for male groups and 2 weeks to 20 weeks for female groups) are shown in Table 1. The results of fitting the model given in the Appendix to the data are shown in Table 2.

Mutated crypts in untreated mice

No mutated (negative) crypts were observed in the 4-week-old control group. One half-crypt, seven singles and one triplet cluster were found in the four mice of the 26-week-old untreated control group. One negative cluster (4 crypts) and one pair of negative crypts two crypts apart were observed in one control mouse at 136 weeks, and five pairs of negative crypts were also observed in the other mice. Thus negative crypts accumulate with age at a rate of about 0.1 per 10^5 crypts per week in control untreated animals (~0.35 per 10^5 crypts per month). About 2 crypts/10^5 crypts per mouse underwent fission by 136 weeks (column J in Table 1). Fitting the data to equations (1) suggests a crypt cycle of 187±44 weeks for these untreated mice (Fig. 3A). This suggests that about only a third of the crypts divide once in the life of a laboratory mouse (3 years).

Mutations after NEU or MNNG

There was considerable mouse-to-mouse variability in parts of the experiments for reasons that are unclear but may reflect differential dose distribution within the gut or differential metabolism.

The data however indicate the following.

(1) The mutagens induce many mutated crypts and these take several weeks to become fully expressed as monophenotypic crypts. This conversion time is about 7±2 weeks for MNNG and somewhat longer at 12±3 weeks for NEU.

(2) There are some large clusters present 16 and 52 weeks after MNNG treatment but the variability between mice and the small group size make it difficult to conclude much from these data. Abnormally large numbers of single mutated crypts at 16 weeks in the male treated group were seen and there is no explanation at present for this (Fig. 3C).

(3) There are mixed crypts at late times after MNNG, suggesting that some of the agent, or some effect from the agent, is persisting and generating new mutations at late times. The data as a whole are summarised in Table 1 and Fig. 4.

(4) MNNG is causing some crypt sterilisation (Fig. 5). At the dose used (50 mg/kg) about 20% of the crypts are killed.

DISCUSSION

We have shown that the crypts in the small intestine can ultimately be derived from a single mutated stem cell, since most mutated crypts are homogeneously negative 2 weeks after

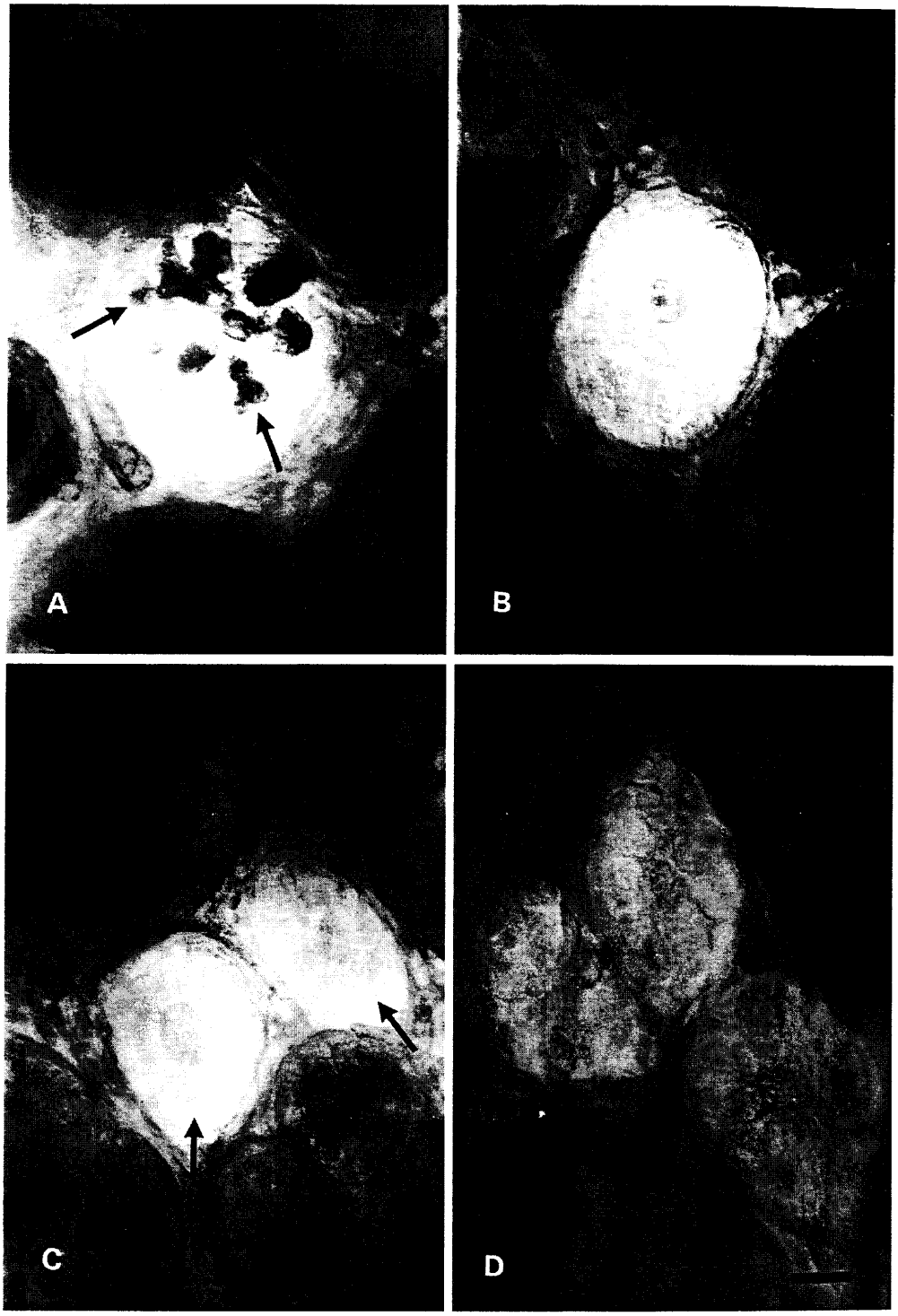

Fig. 1. DBA-Px negative staining crypts in the small intestine after NEU or MNNG treatment. (A) Twelve DBA-Px positive Paneth cells in an otherwise negative staining crypt (arrows). (B) Single or isolated DBA-Px-negative staining crypts showing positive staining crypts, and positive staining blood vessels are adjacent to the DBA-Px negative crypt. (C) A pair of adjacent negative staining crypts. (D) A triplet of negative staining crypts. Bar, 20 μm.

mutagen treatment and virtually all mixed crypts (half-negative) are lost by 16 weeks. The large number of mixed crypts in female mice at 20 weeks after MNNG is surprising and there is no explanation at present (see Results). The precise timing of the conversion from mixed to monophenotypic and the mechanism involved remain uncertain. We believe that each crypt is unlikely to have a single slow-cycling ultimate stem cell (Winton and Ponder, 1990). It is more likely that there is a stochastic replacement of stem cells, which ultimately generates monoclonality from one mutated stem cell amongst several non-mutated stem cells (Loeffler and Grossmann, 1991; Loeffler et al., 1993). This process may be speeded up if some stem cells are injured and stem cell regeneration is required (MNNG is faster than NEU, i.e. 12 weeks→7 weeks). We have demonstrated that these doses of mutagens result in significant levels of cell death (apoptosis) particularly in the stem cell region (Li et al., 1992). The increased incidence of clustered negative crypts over relatively short times compared with the controls further suggests that significant stem cell damage has been induced. In fact it suggests that the level of damage is such that about 20% of the crypts are sterilised. Paneth cell-positive but otherwise negative crypts and half-crypts probably represent intermediate stages during the development of uniform negative staining

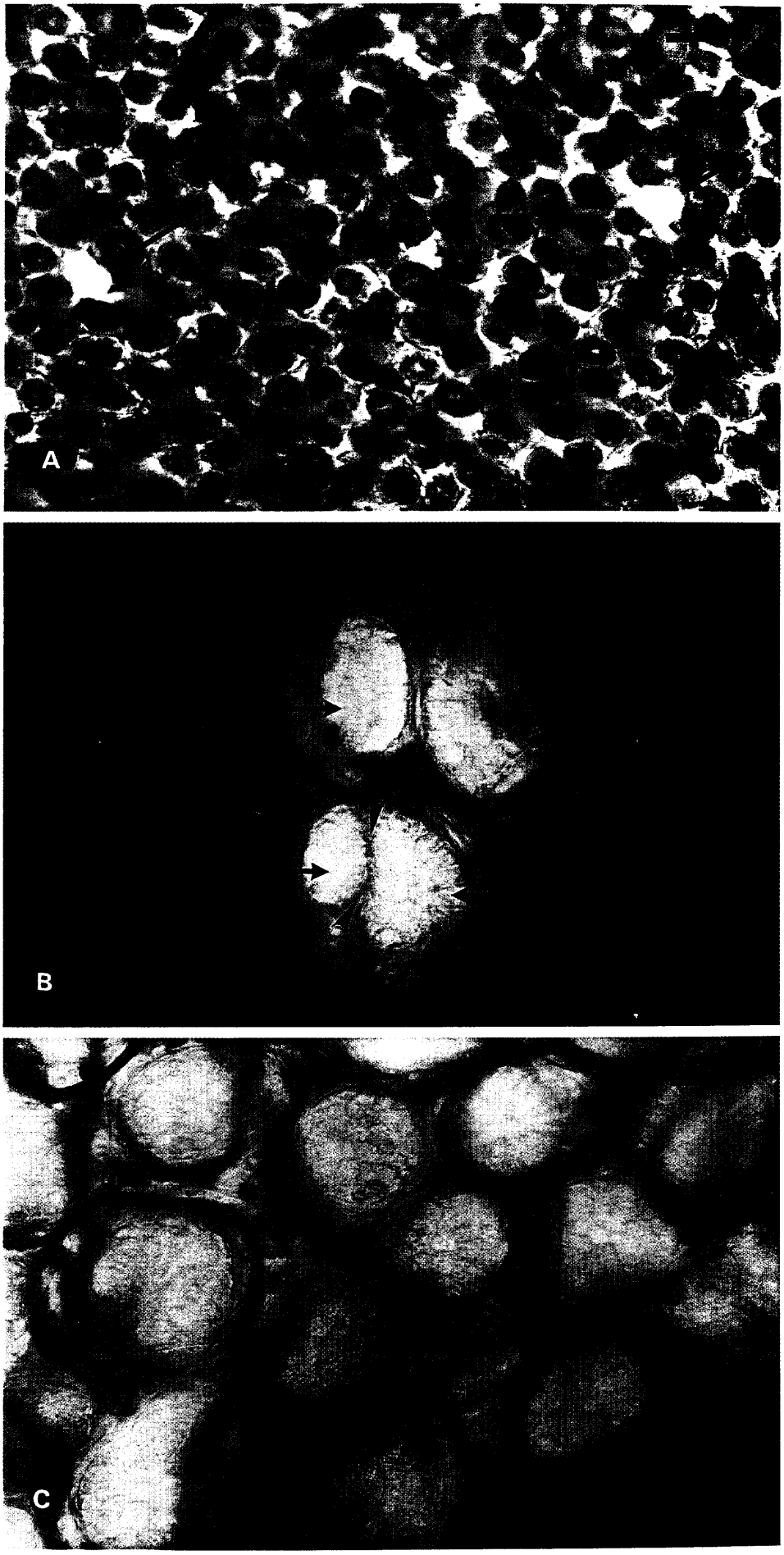

Fig. 2. The small intestinal crypts of C57BL/6J × SWR F1 mouse treated with MNNG. (A) Two isolated DBA-Px negative crypts (arrows) amongst many positive staining crypts (bar, 50 μm; see also Fig. 1B). (B) A cluster of four DBA negative crypts (arrows) showing the probable process of crypt fission (arrowheads). (C) A large cluster of negative staining crypts (37 negative crypts) from a mouse at 16 weeks after MNNG. Many negative crypts appear as white circular areas with the individual crypt cells that are visible. The positive staining blood vessels are adjacent to, or partly overly, the DBA-Px negative crypts. Positive stained crypts are visible in the lower right corner. Bar, 20 μm.

Table 1. Mutagenicity of mutagens on ileal crypts of C57BL/SWR F1 mice

Category Treatment	Total no. of −ve crypts I	No. of single −ve crypts A	No. of pair −ve crypts D	No. of triplet −ve crypts E	2 −ve crypts 1 crypt apart G	2 −ve crypts 2 crypts apart H	Clusters of −ve crypts F	No. of half −ve crypts B	No. of Paneth +ve −ve crypts C	Total no. of crypts budded at least once* J
Controls 4 weeks old	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0	0.0±0.0	0.0±0.0	0.0±0.0
Controls 26 weeks old	2.8±1.4	1.8±0.8	0.0±0.0	0.3±0.3	0.0±0.0	0.0±0.0	0	0.3±0.3	0.0±0.0	0.3±0.3
Controls 48 weeks old	2.5±0.3	2.5±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0	0.0±0.0	0.0±0.0	0.0±0.0
Controls 78 weeks old	8.5±2.7	5.3±1.5	0.5±0.3	0.3±0.3	0.0±0.0	0.0±0.0	1[4]†	0.0±0.0	0.5±0.3	1.0±0.6
Controls 130 weeks old	18.0±4.0	9.3±1.2	2.0±0.7	0.3±0.3	0.0±0.0	0.0±0.0	3[4,2×5]	0.5±0.3	0.0±0.0	3.0±1.1
Controls 136 weeks old	12.3±3.6	8.3±1.7	1.3±0.6	0.0±0.0	0.0±0.0	0.3±0.3	1[4]	0.0±0.0	0.0±0.0	1.9±0.9
2 weeks after NEU, 100 mg/kg	11.5±4.7	2.0±0.6	0.3±0.3	0.3±0.3	0.0±0.0	0.0±0.0	2[2×4]	5.0±2.0	1.3±0.9	1.1±0.6
16 weeks after NEU, 100 mg/kg(3)‡	51.8±9.8	37.5±5.2	2.5±1.0	0.3±0.3	0.0±0.0	1.0±0.7	2[4,16]	0.3±0.3	1.3±0.5	4.5±1.4
52 weeks after NEU, 100 mg/kg(3)	88.3±10.5	62.0±10.4	8.3±1.8	0.7±0.7	1.0±0.6	0.7±0.7	1[7]	0.0±0.0	2.0±1.5	11.0±2.5
86 weeks after NEU, 100 mg/kg(2)	63.5±3.5	34.5±0.5	11.0±2.0	1.5±1.5	0.0±0.0	0.0±0.0	1[4]	0.5±0.5	0.0±0.0	13.0±0.0
2 weeks after (♂) MNNG, 50 mg/kg	74.8±16.1	30.3±7.4	1.0±0.4	0.0±0.0	1.0±0.6	2.0±0.8	0	2.8±1.4	33.8±16.3	4.0±0.9
16 weeks after (♂) MNNG, 50 mg/kg	536.7±46.8	235.7±16.2	57.7±6.8	15.7±1.2	15.0±4.0	11.3±0.7	32[14×4,6×5,3×6,7,10, 12,13,16,2×17,21,37]	0.0±0.0	1.3±1.3	107.2±9.0
52 weeks after (♂) MNNG, 50 mg/kg	359.8±50.8	152.8±29.7	47.7±11.7	15.0±2.2	2.8±1.3	1.0±0.4	47[34×4,8×5, 36,12,17]	0.0±0.0	0.3±0.3	78.5±15.9
2 weeks after (♀)§ MNNG, 50 mg/kg(3)	41.3±27.4	20.7±13.2	2.3±1.9	0.0±0.0	0.3±0.3	1.0±1.0	0	3.7±3.7	9.7±4.4	3.6±3.2
3 weeks after (♀)§ MNNG, 50 mg/kg	93.8±52.5	32.8±16.6	4.5±2.0	1.5±1.5	2.0±1.4	3.3±3.0	1[4]	8.8±2.3	27.2±16.6	11.6±7.9
20 weeks after (♀)§ MNNG, 50 mg/kg(3)	297.3±30.6	185.0±32.8	15.3±1.8	1.3±0.3	5.0±1.2	7.3±0.9	9[7×4,5,6]	36.0±4.0	4.0±2.0	32.0±2.7

All values are corrected to 10^5 crypts; mean ± s.e.m.
*Clusters greater than 20 excluded from total, which are assumed to be spontaneous mutations occurring during development.
†Number of negative crypts/cluster.
‡Number of mice per group; others are 4 mice per group.
§Injected when 52 weeks old for female mice.

(Ponder and Wilkinson, 1983). It is interesting that the frequency of half-negative crypts increased gradually over 18 weeks in the female groups treated with MNNG.

Fission of crypts is thought to be a process invoked when crypt numbers need to be increased during post-natal growth or after injury (Khokhar and Potten, 1979/80). New crypts have been assumed to be derived by a longitudinal fission of pre-existing crypts through a median plane (Maskens and Dujardin-Loits, 1981) and crypts can be seen that appear to be splitting from the base upwards (Cairnie and Millen, 1975), i.e. the initial steps start in the stem cell zone (Potten and Hendry, 1983). Clustered negative crypts could be the consequence of repeated binary crypt fission or the simultaneous splitting of crypts into several new crypts (Cairnie and Millen, 1975).

The large clusters observed may be the consequence of mutations in crypts early in gut development (the sampling was too small to see them at all times) or the consequence of local crypt destruction with crypt regeneration arising from a neigh-

Table 2. The summary of fitting the experimental model

Treatment	Initial mutation number M_0	Mutation rate (week) α_m	Conversion rate (week) α_c	Fission rate (week) α_f
Controls	—	0.080±0.005	—	0.0037±0.0009
NEU	65±7	—	0.059±0.016	0.0047±0.0014
MNNG	1700±9500	—	0.007±0.042	0.016±0.008
MNNG (♀)	265±32	—	0.134±0.059	0.011±0.003
MNNG (♂) (♀+♂ pooled)	265±29	—	0.094±0.026	0.012±0.002

Values are mean ± s.e.m. Data were corrected to 10^5 crypts)

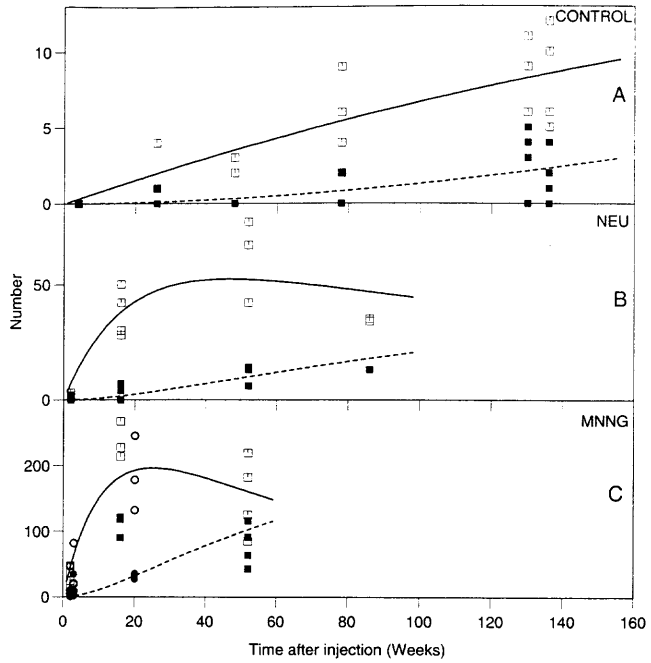

Fig. 3. Numbers of single monoclonal crypts (open symbols, continuous lines) and clusters of monoclonal crypts (filled symbols, broken lines) as a function of time after injection (treated mice) or with age (control mice). (A) Control mice; (B) NEU-treated mice; (C) MNNG-treated mice. Male mice are shown as squares (□, ■) and female mice as circles (○, ●). The lines represent the fitted values using the model given in the Appendix and the values in Table 2.

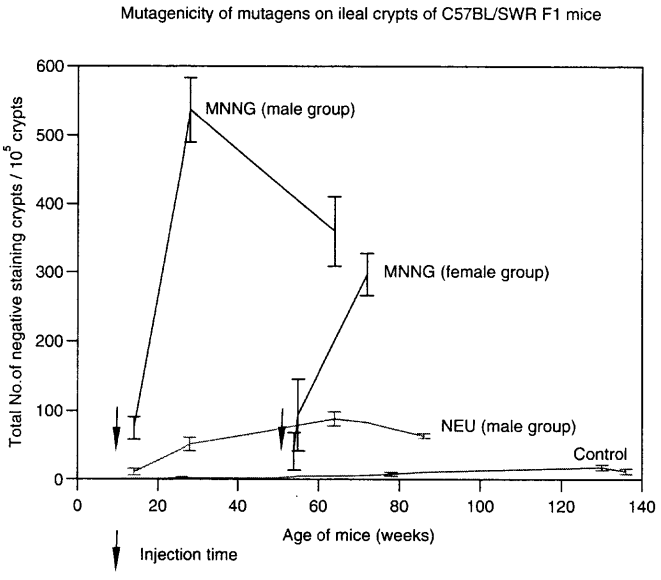

Fig. 4. The incidence of *Dlb-1* mutated crypts in whole-mounts of small intestinal epithelium from MNNG- or NEU-treated F1 mice. The values shown are inclusive of all the categories of the mutated crypts described in the Results and shown in Table 1, each point represents the total number of DBA-negative crypts (mean ± s.e.m) derived from 3 or 4 mice. MNNG (50 mg/kg) or NEU (100 mg/kg) was injected intraperitoneally into 12-week-old male (MNNG, NEU) or 52-week-old female mice (MNNG). The injection times for each group are shown as arrows in the figure.

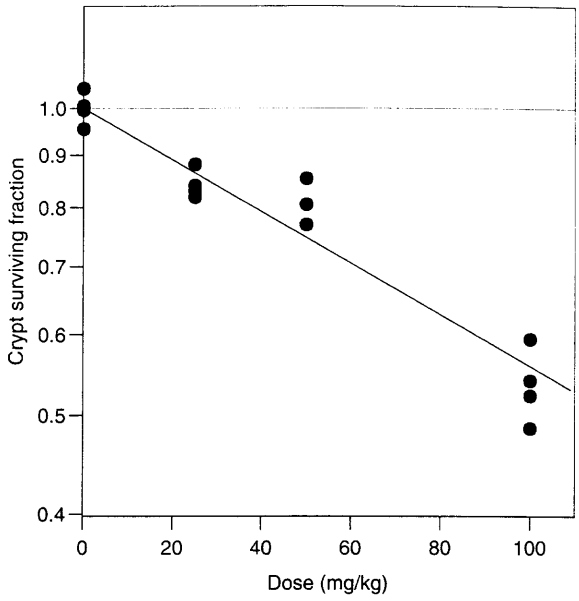

Fig. 5. Dose-survival curves for the ileal crypts of C57BL/6J × SWR mice after MNNG treatment using the crypt microcolony assay. The data are shown as the surviving fraction of ileal crypts versus dose of MNNG. Each point represents the survival of an individual mouse corrected for changes in crypt size. The line shows a fit to the data with D_0=172 mg/kg. The D_0 is the mean lethal dose reducing survival from f to fe^{-1}. It is the reciprocal of the slope of the line.

bouring surviving mutated crypt. We have shown that there is a crypt cycle because there is evidence of some clusters late in life but the cycle we estimate is 10 times longer than that proposed earlier (Bjerknes, 1986; Totafurno et al., 1987). This difference may be in part for methodological reasons. Bjerknes observed the fate of 328 crypts over a period of 3 weeks by video recording of crypts seen through the muscularis mucosa, and 49 of the crypts were found to have divided within that period. A complication here may be the fact that, besides binary fission, crypts may undergo multiple fission, splitting into many crypts simultaneously. We have seen multiple fission in the normal mouse, which confirmed an earlier observation by Cairnie and Millen (1975) in the irradiated mouse. We have noticed that crypt fission in normal mice is a rare event, i.e. there were about three clusters, suggesting budding in 100,000 crypts over 130 weeks. Only occasional crypt fission suggests a long crypt cycle time. However, our calculation may be an underestimate because we do not measure the life span of a newborn mutated crypt. If newly formed crypts are lost or move apart this would reduce the estimate, but this seems unlikely. The difference between the calculation of Bjerknes' and ours might also be influenced by differences in mouse strain. The number of crypt fissions observed were certainly different in the two cases.

The data for mutations in the control mice would suggest a spontaneous somatic cell mutation rate of 0.080±0.005 per 10^5 crypts per week. Any crypt fission in an adult mouse might compensate for any crypt loss or the continual slight growth of the gut with age (Potten and Hendry, 1983). Crypt loss is difficult to observe but there was no significant variation in the total number of crypts per grid between the different-aged untreated mice (data not shown).

Denitrosation of MNNG is affected by enzymes occurring in the stomach, liver and kidney (WHO Publications, 1979) and MNNG might accumulate in the organs, after injection, for some time and be released from the organs later. This might explain the late appearance of mixed crypts. NEU mainly produces chromatid and chromosome aberrations, single chromatid and isochromatid breaks, exchanges and multiple breaks and is rapidly lost from the body (WHO Publications, 1978). In contrast, denitrosation of MNNG is affected by enzymes occurring in the tissues (WHO Publications, 1979), and it is retained in the body (biological half-life) longer than NEU. Thus, MNNG exerts its mutagenic effects continuously over a longer time. This may be the explanation, at least in part, for the fact that MNNG is more mutagenic and cytotoxic than NEU.

The presence of some large clusters at late times after MNNG suggests that tissue regeneration processes may be occurring as a result of the sterilisation of some crypts. Such observations would suggest that the tissue somehow recognises that crypts are absent and signals surviving crypts to compensate for the loss. Other studies have indicated that similar compensatory increases in stem cell proliferation within crypt occurs after even small levels of cytotoxic damage (Potten, 1991a,b), and modelling studies have suggested that the stem cells effectively regulate their own numbers after perturbation (Paulus et al., 1992).

APPENDIX

The model

(1) Normal crypts are converted to mutated (or mixed) crypts (M) at a constant rate α_m. There may be some mutated (mixed) crypts at time zero (M_0). Only those crypts that are going to be converted to monoclonal mutant crypts are counted in M and M_0.

(2) These mixed crypts (M) are converted to monoclonality (S) at a constant rate α_c.

(3) Monoclonal crypts (S) divide at a rate α_f to produce clusters (C).

(4) Any complications such as the fission of mixed crypts are ignored. The numbers of mutants are assumed to be small.

The following differential equations then describe the model:

$$dM(t)/dt = \alpha_m - \alpha_c M(t)$$

$$dS(t)/dt = \alpha_c M(t) - \alpha_f S(t)$$

$$dC(t)/dt = \alpha_f S(t)$$

with initial conditions $M(0)=M_0$; $S(0)=S_0$ and $C(0)=C_0$; t, time.

These can be readily solved in general, but here we are specifically interested in two limiting cases, there being insufficient data to fit the full model.

(1) Control animals have very low numbers of initial mutations and a very low mutation rate. Fitting $M_0=S_0=C_0=0$ and assuming $\alpha_m << \alpha_c$, the equations then simplify to give:

$$M(t) = 0$$

$$S(t) = (\alpha_m/\alpha_f)\,(1-\exp(-\alpha_f t))$$

$$C(t) = \alpha_m t + (\alpha_m/\alpha_f)\,(\exp(-\alpha_f t)-1).$$

(2) Immediately after treatment with a mutagen there will be high numbers of mutations but few converted crypts. After the initial treatment the number of new mutations will be very low compared with the total number present, and effectively undetectable. Fitting $S_0=C_0=0$ and $\alpha_m=0$, the equations again simplify to give:

$$M(t) = M_0 \exp(-\alpha_c t)$$

$$S(t) = M_0\,\alpha_c\,(\exp(-\alpha_f t)-\exp(-\alpha_c t))/(\alpha_c-\alpha_f)$$

$$C(t) = M_0\,\{1-(\alpha_c \exp(-\alpha_f t)-\alpha_f \exp(-\alpha_c t))/(\alpha_c-\alpha_f)\}.$$

Note that the number of observed mixed crypts does not correspond to the number M in the model. However, the two measures would show the same sort of patterns if all mixed crypts could be identified. As it is more likely that only crypts far from monoclonality (i.e. 50/50 mutant cells rather than nearly all mutant or very few mutant cells) will be identified as such, the number observed will have a very complex and dynamic relationship to the number present, and the data are not capable of revealing anything more than that mixed crypts are seen in the mutated cases, but not in controls where $M \approx 0$ is predicted.

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